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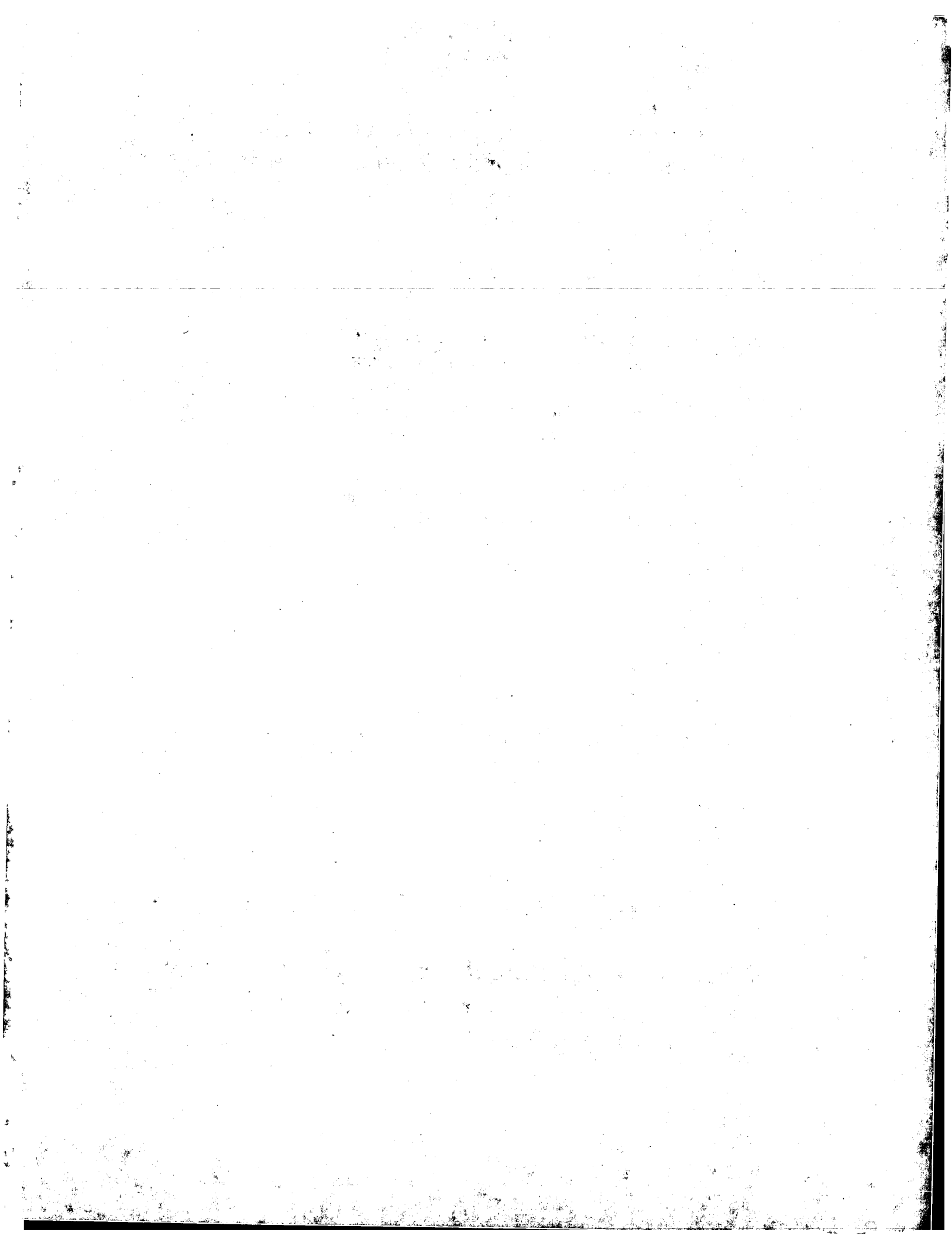
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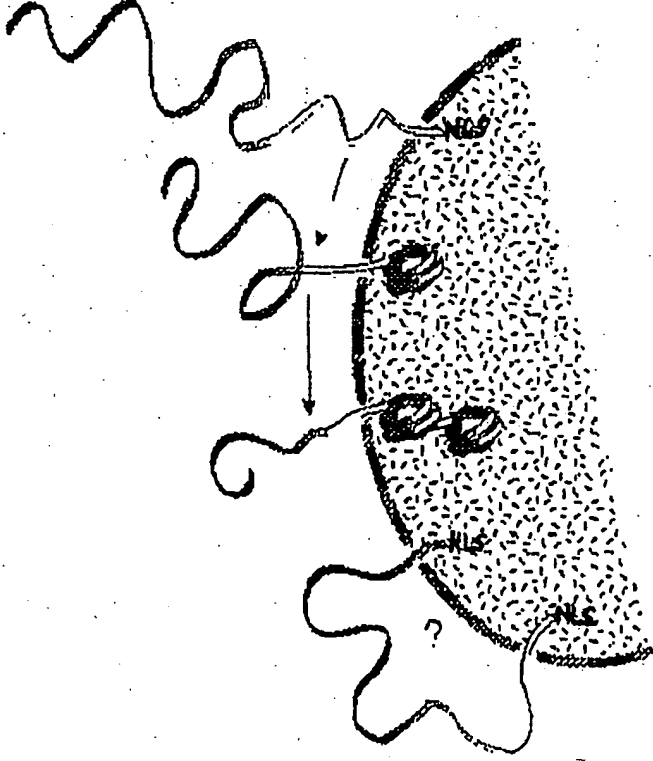
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<p>(54) Title: NON-VIRAL TRANSFECTION VECTOR</p> <p>(57) Abstract</p> <p>A non-viral transfection vector which is to be delivered into the nucleus of a cell comprises a DNA molecule that is equipped with 1 to 25 NLS conjugates. Each conjugate comprises a nuclear localization signal (NLS) covalently linked to an oligonucleotide. The NLS conjugate may be covalently linked to one or both termini of a linear DNA molecule, associated with a plasmid DNA molecule by forming a triple helix, or inserted in a plasmid DNA molecule by strand invasion. The transfection vector is useful for gene therapy applications.</p> 		

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Non-viral transfection vector

The present invention relates to the field of non-viral gene delivery.

5 Translocation of exogeneous DNA through the nuclear membrane has been shown to be a major problem in gene delivery technologies. The nuclear membrane of eukaryotic cells is freely permeable to solutes of size up to ca. 9 nm (e.g. 40-60 kDa proteins). Transport of
10 larger molecules through nuclear pores is signal-mediated, involves shuttle molecules and requires energy. The basic peptide derived from the SV40 large T antigen (PKKKRKV) is a nuclear localization signal (NLS) that mediates binding of the karyophilic protein
15 to importin- α (Adam and Gerace, 1991).

Complex formation triggers binding to importin- β and the ternary complex is then carried through the nuclear pore with the help of the GTPase Ran. Macromolecules and particles up to 25 nm have been shown to enter the
20 nucleus in this way, although the translocation mechanism is still not fully understood (Panté and Aebi, 1995; Nigg, 1997; Ohno et al., 1998; Melchior and Gerace, 1998).

Eukaryotic DNA viruses which replicate in the nucleus
25 seem to be capable of diverting the cell's nuclear import machinery to their own benefit (Greber et al., 1997; Greber, 1998). Recombinant viruses derived therefrom are being used to carry therapeutic genes.

into humans. However, the host's immune response is currently a limitation to the clinical development of viral gene therapy. Nonviral alternatives using plasmid DNA and cationic carrier molecules suffer from a different drawback, the low efficacy of gene delivery. The main barrier to transgene expression *in vitro*, is the nuclear membrane (Zabner et al., 1995; Labat-Moleur et al., 1996; Hagstrom et al. 1997). Since breakdown of this membrane during cell division helps nuclear localization, this obstacle is probably still more a problem *in vivo*, where cells can be considered as resting with respect to the lifetime of DNA.

Several attempts to improve entry of plasmid DNA into the nucleus have been suggested. These include use of electrostatic binding of DNA to cationic NLS-containing proteins (Kaneda et al., 1989; Fritz et al., 1996) or peptides (Collas et al., 1996) or lipids (Remy et al., 1995)), as well as sequence-specific binding of DNA to karyophilic proteins (Fominaya, J. & Wels, W. 1996; Langle-Rouault et al., 1998). Yet such "piggyback" nuclear transport relies on the unpredictable stability of the complexes within the cytoplasm. Recent work by the group of Jon Wolff (Sebestyén et al., 1998) with digitonin-permeabilized cells demonstrated nuclear accumulation of fluorescently-labelled DNA that was randomly tagged with hundreds of NLS peptides covalently attached to the DNA; nuclei of intact cells did not take up the modified DNA.

It was an object of the present invention to overcome the problems of the previous methods of nuclear

transport of DNA and to provide an improved gene delivery system.

The present invention is directed to a non-viral transfection vector comprising a DNA molecule which is
5 to be delivered into the nucleus of a cell, characterized that said DNA molecule is equipped with 1 to 25 conjugates comprising a nuclear localization signal covalently linked to an oligonucleotide, each of which NLS-conjugate has been

- 10 i) covalently linked to one or both termini of a linear DNA molecule, or
- ii) associated with a plasmid DNA molecule by forming a triple helix, or
- 15 iii) inserted in a plasmid DNA molecule by strand invasion.

In the following, the nuclear localization signal is termed "NLS".

NLSS are peptides which are known to be required for nuclear transport of karyophilic proteins. They are
20 part of the primary structure of most nuclear proteins and contain one or two clusters of basic amino acids. Typically, they are six to eighteen amino acids in length.

A wide range of NLSS from various organisms has been
25 characterized, examples of NLSS that can be used in the present invention are given in WO 95/31557, which is incorporated herein by reference in its entirety.

The NLS must fulfil the requirements to direct the DNA or the protein encoded by the DNA, respectively, to the nucleus.

Depending on the organism whose cells are to be
5 transfected, e.g. an animal or a plant, the NLS is selected from a karyophilic protein expressed by that organism or from a virus infectious for that organism.

A preferred NLS used in the present invention is the NLS of the SV40 virus large T antigen.

- 10 The NLS peptide can be synthesized according to standard methods of peptide chemistry. In general, the peptide is identical to the naturally occurring NLS. However, if desired, e.g. to improve the nuclear transporting function of the peptide, the peptide may
15 be modified, e.g. by replacing one or more of the basic amino acids by other basic amino acids. Another NLS modification, which has been used in the experiments of the present invention, is the extension of the NLS beyond its actual nuclear localization domain by the
20 N-terminal amino acids of a karyophilic protein, preferably the authentic protein that the NLS originates from. The peptide may carry an amino acid such as cysteine, lysine, tyrosine, threonine, serine that allows convenient linkage of an oligonucleotide.
- 25 Generally, the length of the NLS is six to sixty, preferably six to forty, even more preferably six to thirty amino acids.

In order to assess whether a selected NLS is suitable for the cell to be transfected, i.e. that it directs

the protein of interest into the nucleus, assays are conducted which are known in the art, e.g. those described by Garcia-Bustos et al., 1991; Sandler et al., 1989; Citovsky et al., 1992; Sebestyén et al., 1998). These assays are based on the detection of the protein in the nucleus, e.g. by histochemical methods. A useful assay involves the use of a reporter gene, e.g. the luciferase gene; the delivery of the luciferase is detected by standard luciferase detection methods as described in the Examples of the present invention.

Preferably, the NLS contains a reactive thiol group from a cysteine residue and is conjugated to the oligonucleotide by conventional methods, e.g. by amino-modifying the oligonucleotide and linking the two compounds with bifunctional cross linkers like SMCC (4-(N-maleimidomethyl)-cyclohexane-1-carboxylic acid N-hydroxysuccinimide ester).

The amino-modified oligonucleotide can also be linked to the thiol containing peptide by using N-[(iodoacetyl)oxy]succinimide, N-[(bromoacetyl)oxy]succinimide, iodoacetic anhydride or bromoacetic anhydride as crosslinking reagents (Reed et al., 1995, Wei et al., 1994).

Alternative coupling methods that can be used employ an N-(bromoacetyl)peptide (or an N-(iodoacetyl)peptide) that can react with a thiol containing oligonucleotide as described by Arar et al., 1995. Other coupling strategies that may be used are those described by de La Torre et al., 1999, i. e. reacting a thiol-containing oligonucleotide with a maleimido-derivatized

peptide, or the direct synthesis of the peptide and oligonucleotide on the same support, yielding a oligonucleotide-3'-peptide conjugate.

Alternative coupling methods that can be used in the present invention, e.g. activation of the 5'-phosphate groups of the oligonucleotide with imidazole or tetrazole in the presence of a carbodiimide compound and subsequent displacement of that group by the terminal amino group of the NLS, are disclosed in WO 88/05077, which is incorporated herein by reference in its entirety.

Preferably, the DNA molecule is equipped with 1 to 20, more preferably with 1 to 10, in particular with 1 to 5 NLS conjugates.

In the embodiment i) of the present invention, the DNA molecule is linear.

The peptide-oligonucleotide has a sequence that allows to form a hairpin and has, preferably at its 5'-end, a cohesive extension that is ligated, by standard cloning methods, to a complementary cohesive sequence generated at the end of the DNA molecule. The linear DNA molecule carries one or two, preferably one, NLS(s) covalently linked to the hairpin.

This hairpin-forming oligonucleotide is preferably 15 to 70 nucleotides in length. The NLS is conjugated to the oligonucleotide by coupling the peptide nucleotide modified with a reactive group, e.g. a linker containing an amino group or a mercapto group,

or by another coupling method as described above, in the loop of the hairpin.

Preferably, in the embodiment i) of the invention, only one terminus of the linear DNA molecule carries an oligonucleotide hairpin which has an NLS coupled to it,
5 the other terminus of the DNA molecule is capped with an identical or different oligonucleotide hairpin that does not carry an NLS. In the experiments of the present invention, it has been surprisingly shown that
10 a single NLS is sufficient to carry DNA to the nucleus.

Figure 1 depicts an example for the strategy for the preparation of a dsDNA fragment coupled to an NLS peptide. A functional luciferase gene of 3380 bp was cut out of pCMVLuc with *XmaI* and *SalI*. Further
15 digestion with *XmnI* and *BspHI* cut the unwanted restriction fragment into small fragments (970, 875, 768 and 240 bp) which were removed by sucrose gradient centrifugation. The capped CMVLuc-NLS DNA was obtained by ligation of the ³²P radioactive (*) oligo-peptide and
20 oligo-cap hairpins to the restriction fragment.

The following considerations have lead to the design of the nuclear transport system according to embodiment i) of the invention:

When fully counterion-condensed, a single plasmid
25 molecule collapses into a sphere of ca. 25 nm (Blessing et al., 1998)) which is already at the size-exclusion limit for signal-mediated nuclear import (Ohno et al., 1998; Görlich and Mattaj, 1996). Plasmid condensation with cationic lipids or polymers generally leads to
30 even larger, multimolecular aggregates which reach the

cytoplasm after binding to cell-surface anionic proteoglycans (Labat-Moleur et al., 1996; Mislick and Baldeschwieler, 1996) and eventual escape from the formed vacuoles (Zabner et al., 1995; Plank et al., 1994; Boussif et al., 1995). Since the particles are too large to cross an intact nuclear membrane, transfection of resting cells can reasonably only be accounted for by uncomplexed DNA that has been released by exchange with phosphatidylserine (Xu and Szoka, 1996) or heparan sulfate (Labat-Moleur et al., 1996) present in the vacuolar membrane. Any molecular information (e.g. NLS) borne by the cationic vector will thus be lost prior to reaching the nuclear membrane, hence its ineffectiveness.

15 In sharp contrast to a condensed DNA molecule, a free hydrated DNA double helix (3 nm) is thin enough to enter the nuclear pore by diffusion, i.e. with no energy nor signal requirement. Restricted intracellular motion of DNA (the cytoplasm is equivalent to 13% dextran (Luby-Phelps, 1994)) and a rather low nuclear pore coverage (<10% of the membrane surface (Maul and Deaven, 1977)) however give this event a low probability to occur: the nuclear membrane has been recognized as a major barrier to gene delivery (Zabner et al., 1995; Labat-Moleur et al., 1996). Quantitative cytoplasmic microinjection of DNA (Pollard et al., 1998; Mirzayans et al., 1992; Dowty et al., 1995) indeed led to less than 0.1% of the DNA being expressed. The probability for DNA to find and subsequently enter a nuclear pore can be increased by a bound karyophilic signal peptide able to dock DNA to the nuclear pore filaments and help an initial part of the molecule to

cross the membrane. The DNA-karyophilic signal link needs however be stable, therefore the peptide was chemically linked to the oligonucleotide.

The linear DNA molecule approach i), which is
5 exemplified in the experiments of the invention, takes advantage of a chemically controlled pathway to irreversibly link an NLS-peptide to one end of a gene. In the experiments of the inventions, in contrast to the state of the art, the level of transgene expression
10 following transfection was chosen as a test of success.

The major achievement of the linear DNA/NLS approach is that, for the first time using a nonviral technology, the amount of DNA required to effectively transfect cells in vitro has shifted from the microgram to the
15 nanogram range. Although the improvement (1-3 orders of magnitude) varies with cell type (showing that the nuclear membrane is not the only possible barrier), even the modest enhancements observed for primary cell cultures look interesting (1000%) when not presented on
20 a logarithmic scale. To show that this result was a consequence of the involvement of the nuclear import machinery, a Lys \Rightarrow Thr mutation was used which indeed abolished the beneficial effect brought about by the DNA-bound NLS sequence.

25 Taking the raised hypothesis further, the last unanswered question deals with threading of the rest of the plasmid molecule (Figure 7). Random-walk diffusion of a micrometer-long molecule would be inefficient and slow. Fortunately, naked DNA will not remain free in
30 the nucleus : histones (and eventually basic nuclear

matrix proteins) indeed quickly assemble transfected DNA into chromatin-like structures (Cereghini and Yaniv, 1984; Jeong and Stein, 1994), thus providing a mechanism for pulling and condensing the filamentous molecule into the nucleus. Following this line of thinking, too many NLS signals distributed along the DNA may actually inhibit nuclear entry if the nucleic acid is longer than the distance separating adjacent pores (Fig. 7). A straightforward calculation using the pore density in HeLa cells (Maul and Deaven, 1977) shows this to become probable above 1 kbp.

In embodiment ii) of the invention, the DNA molecule is a plasmid encoding any protein of interest, and the NLS peptide is covalently linked to an oligonucleotide that recognizes a homopurine/homopyrimidine sequence within the plasmid in a sequence specific manner (Le Doan et al., 1987, Moser and Dervan, 1987). The interaction of the oligonucleotide with the duplex DNA takes place in the major groove leading to a local triple helix structure (Fig. 8). The use of a triple-helix structure to obtain strong association between a duplex molecule and an oligonucleotide is an attractive method to generate the NLS-bearing plasmid molecule.

Two families of DNA triple helices have been characterized that differ in their third-strand sequence composition and relative orientation. In the pyrimidine family (Py*Pu-Py), a homopyrimidine strand (Py) is bound (*) parallel to the purine strand of target duplex through Hoogsteen base pairing. In the purine family (Pu*Pu-Py), a homopurine strand (Pu) is bound in an anti-parallel orientation to the purine

strand of target duplex through reverse Hoogsteen base pairing. In the purine family, it has been shown that G-rich homopurine oligonucleotides are able to form triple helices with high stability compatible with physiological conditions (Debin et al., 1997, Nakanishi et al., 1998). This type of sequence, which is preferred in this embodiment of the invention, leads to a stable non-covalent association between the NLS-oligonucleotide and the plasmid (Fig. 9).

10 The selected homopurine-homopyrimidine oligonucleotide sequence can be introduced in the plasmid by conventional cloning methods, e.g. as described in Sambrook et al., 1989, at a specific position outside of the promoter region, outside of the gene coding for the protein of interest, and of the polyadenylation
15 signal. Preferably, the oligonucleotide, which in this embodiment of the invention is a triple-helix-forming oligonucleotide (TFO), is between 8 and 40 nucleotides long. Some examples of G-rich sequences, that are
20 useful for this embodiment of the invention, e.g. a nearly perfect 45mer polypurine tract localized in the gag gene of Friend murine leukemia virus (F-MuLV), are presented in Fig. 10 (Debin et al., 1997; Nakanishi et al., 1998; Svinarchuk et al., 1994; Rando et al.,
25 1994). Another G-rich sequence that may be used in the present invention, was described by Sedelnikova et al., 1999; it comprises 39 nucleotides and allows efficient triple-helix interaction with a homopurine-homopyrimidine containing plasmid.

30 It has been reported for several G-rich homoPu sequences that triple helix association can persist

inside cells for 1 to 3 days (Debin, et al., 1997, Svinarchuk, et al., 1994). Therefore, for appropriate homoPu-homo-Py sequences, the NLS-oligo/plasmid triplexes are expected to be stable enough to remain
5 undissociated inside the cell until the triplex is carried to the nucleus. Furthermore, by introducing several identical homoPu-homo-Py sequences in the plasmid, the influence of both number and distance between NLS's on nuclear import can be assessed in
10 order to optimize the design of the construct.

In an embodiment of the invention, the oligonucleotide is chemically modified in order to increase the stability of the oligonucleotide and the interaction with the plasmid. The oligonucleotides may contain
15 modifications of the intra-nucleotide linkages such as phosphorothioate, methylphosphonate, or N3-P5 phosphoramidite modifications. Modifications of the sugar residues (such as 2'-O-methyl) and modifications of the bases can also be introduced. Modifications of
20 this type have been described by DeMesmaeker et al., 1995; Uhlmann and Peyman, 1990; Iyer et al., 1999.

An alternative modification that may be advantageously used, in particular if small quantities of NLS-plasmid are required, is the so-called "padlock" approach, as
25 described by Escude et al., 1999.

Furthermore, intercalating agents may be used for stabilization of the NLS-oligonucleotide, e.g. as described by Escude et al., 1998.

There are no limitations with regard to the location of
30 the modification in the oligonucleotide.

The NLS peptide is covalently linked to the TFO purine oligonucleotide either at the 5' or at the 3' end of the oligonucleotide according to known methods, as described above for embodiment i). Preparation of the plasmid carrying the NLS peptide(s) simply requires
5 mixing of the NLS-oligonucleotide and the homoPu-homoPy plasmid in appropriate buffer conditions, e.g. 20 mM Tris-acetate pH 7.5, 5-10 mM Mg^{2+} , no K^+ ions.

In the embodiment iii) of the invention, the
10 NLS-oligonucleotide conjugate is inserted into the plasmid DNA by a mechanism that has been termed "strand-invading mechanism". This mechanism has been proposed to explain the binding mode of "peptide
nucleic acids" (PNA) to DNA (Nielsen et al., 1991). It
15 has been described as the strand invasion of a double-stranded DNA by an oligonucleotide (or a PNA) that leads to complementary binding between the invading molecule and the duplex through Watson-Crick
interactions. Local separation of the DNA strands is
20 required and the final conformation is thought to be a D-loop structure.

In a preferred embodiment of variant iii) of the invention, the oligonucleotide is chemically modified, thus displacement of one DNA strand is achieved by the
25 chemically modified oligonucleotide.

The chemical modification allows stabilization of the D-loop structure over the native duplex. As described by Schmid and Behr, 1995, invasion has been observed with synthetic oligonucleotides containing two spermine
30 residues linked to the C2 position of inosine

(Fig. 11). Stabilization of the D-loop structure is provided by spermine modifications that lie in the minor groove and clip both strands together through interstrand hydrogen bonding between each ammonium
5 group and the nucleic bases.

Stabilization of the D-loop structure may be achieved by modifying the oligonucleotide with polycations such as spermine, spermidine, polybasic aminoacids or other polyamines such as $(\text{NH}-(\text{CH}_2)_n)_m\text{-NH}_2$. The oligonucleotides
10 are preferably 10 to 100 bases in length and contain 2 to 15 polyamines; e.g. six spermine molecules on guanine residues.

Since the strand invading mechanism is not restricted to a particular class of sequences, embodiment iii) of
15 the invention does not require providing specific DNA sequences when constructing the plasmid. However, the oligonucleotide requires a number of bases that allow the coupling of the respective number of polyamine molecules, e.g. in the case of modifying the
20 oligonucleotide with two spermine molecules the presence of at least two guanine bases for the chemical coupling of two spermine residues and the chemical coupling of the NLS peptide to the spermine-oligonucleotide will be useful.

25 The chemical modification of the oligonucleotide with the polyamine may be carried out by known methods, described by (Schmid and Behr, 1995).

Furthermore, coupling of the polyamine may also be done either on the 3' or on the 5' end of the
30 oligonucleotide by methods analogous to the methods

described for embodiment ii); in particular those described by Arar, et al., 1995; de La Torre, et al., 1999.

Alternative modifications of the oligonucleotide that
5 can be introduced are those described above for the embodiment ii).

Embodiment iii) of the invention is schematically depicted in Fig. 12.

In general, the DNA molecule encodes a protein of
10 interest to be expressed in an animal or plant cell. Alternatively, the DNA molecule may encode an inhibiting RNA molecule, e.g. an antisense RNA. In view of gene therapy applications in humans or animals, the protein of interest is a therapeutically active
15 protein. Non limiting examples of DNA molecules are given in WO 93/07283. In addition to the coding sequence, the DNA contains regulatory sequences necessary for its expression in the cell; in the case of a plasmid, additional sequences, e.g. encoding
20 selection markers, may be present. There are no limitations as to the sequence of the DNA. The size of the DNA is preferably in a range typical for expression cassettes that are used for expressing eukaryotic genes, i.e. 300 bp in the case of small DNAs like
25 antisense or ribozyme encoding DNA molecules, to 1Mbp, in the case of artificial chromosomes.

Thus, in a further aspect, the invention relates to a pharmaceutical composition which contains, as an active ingredient, the transfection vector of the invention,
30 wherein the DNA encodes a therapeutically active

protein or an inhibiting RNA molecule. For preparing the composition, any inert pharmaceutically acceptable carrier may be used, such as saline, or phosphate-buffered saline, or any such carrier in which the transfection vectors have suitable solubility properties. Reference is made to Remington's Pharmaceutical Sciences, Mack Publishing Co., Easton, PA, Osol (ed.) (1995) for methods of formulating pharmaceutical compositions.

10 In a further aspect, the invention relates to a method for transfecting cells with a DNA molecule, wherein the cells are contacted with the transfection vector of the invention.

Transfection of the cells with the NLS-conjugate-
15 modified DNA molecules of the invention may be carried out by any standard gene delivery technology, e.g. applying them as such, in a suitable physiological solution, by methods known for the delivery of "naked" DNA, or by mixing the DNA containing the NLS-conjugate
20 with an organic cationic transfection vehicle, e.g. a cationic lipid, like the commercially available Transfectam, or so-called "lipoplexes" (commercially available cationic lipids employed for lipofection as described by Felgner et al., 1987, and reviewed by
25 Clark and Hersch, 1999), or with cationic lipids as described by Behr, 1994 or Ledley, 1996, or with the lipid transfection particles described in WO 99/29349. Alternatively, cationic polymers, e.g. polylysine or polyethyleneimine, which may contain a cellular
30 targeting function and/or an endosomolytic function, may be used for delivering the transfer vectors of the invention. Suitable methods are described in

WO 93/07283, or in reviews by Ledley, 1995; Cotten and Wagner, 1993; Boussif et al., 1995.

In the case of delivery of an NLS-equipped plasmid into the cell, a cationic compound may eventually favour the
5 encounter between the plasmid and oligonucleotide sequences. In these particles, both NLS-oligonucleotides and plasmid DNA partners will be protected from nucleases.

The cells may be transfected with the NLS-modified DNA
10 of the invention *in vitro*, *ex vivo* or *in vivo*. Examples for *in vivo* applications are intramuscular, intraarticular or intradermal injection of the tranfection vector or its electroporation, these applications are preferably performed in the
15 absence of gene delivery vehicles like cationic lipids. This also applies for the gene gun-mediated introduction of NLS-DNA.

Brief description of the figures:

- 20 Fig. 1 Strategy for the preparation of a dsDNA fragment
- Fig. 2 Reaction scheme for the chemical coupling steps leading to the oligonucleotide-peptide conjugate (oligonucleotide-NLS)
- 25 Fig. 3 A. Synthesis of the oligonucleotide-NLS
B. The oligonucleotide-NLS conjugate is a substrate of proteinase K

- Fig. 4 The NLS peptide promotes high and sustained transfection levels down to 10 ng DNA
- Fig. 5 Sustained luciferase expression levels are due to the nuclear localization peptide
- 5 Fig. 6 Reporter protein activity appears faster with CMVLuc-NLS
- Fig. 7 Hypothetical scheme of transgenic linear NLS-DNA crossing a nuclear pore
- Fig. 8 Schematic representation of triple-helix interaction
10
- Fig. 9 Non covalent coupling of an NLS peptide to plasmid DNA through triple helix association
- Fig. 10 Some examples of G-rich target sequences for triple-helix formation
- 15 Fig. 11 Interaction through Watson-Crick pairing after strand-invasion of a DNA duplex by a complementary spermine-containing oligonucleotide
- Fig. 12 Non-covalent coupling of an NLS peptide to a plasmid through the strand-invading mechanism
20

Example 1

Synthesis of an oligonucleotide-NLS equipped luciferase gene; purification and proof of structure

i) Chemicals, enzymes and oligonucleotides

4-(N-maleimidomethyl)-cyclohexane-1-carboxylic acid N-hydroxysuccinimide ester (SMCC) was purchased from Sigma. The SAL 34-mer 5' d(TCGATGTCCGCGTTGGCTT
5 XTGCCAACGCGGACA) oligodeoxynucleotide was synthesized by Appligene using an amino-modified deoxythymidine (X; amino-modified dT, Glen Research, USA). The XMA 34-mer 5' d(CCGGCTACCTTGCGAGCTTTTGCTCGCAAGGTAG)
oligodeoxynucleotide, the NLS peptide NH₂-PKKKRKVEDPYC
10 and the mutated-NLS peptide NH₂-PKTKRKVEDPYC with C-terminal amidation were synthesized by Genosys using the standard F-moc chemistry for the solid phase synthesis of peptide. Hairpin structures composed of a
loop of four thymine, a stem of 13 base pairs and a
15 sticky 5'-end were formed by boiling and subsequently cooling the XMA- or the SAL- oligonucleotides in ice. *Xma*I, *Xmn*I, *Sal*I and *Bsp*HI restriction endonucleases, T4 polynucleotide kinase T4 DNA ligase and Exonuclease III were purchased from New England Biolabs (Ozyme,
20 France). Linear 22 kDa (ExGen500) and branched 25 kDa polyethylenimines (PEI) were purchased from Euromedex (Souffelweyersheim, France) and Fluka (Saint-Quentin Fallavier, France), respectively. Transfectam® (dioctadecylamido-glycylspermine, DOGS) was synthesized
25 as described (Behr et al., 1989).

ii) Preparation of the oligo-peptide conjugate

2 OD units (6,46 nmoles, assuming $\epsilon^{260} = 309,800 \text{ M}^{-1}\text{cm}^{-1}$) of the aminomodified SAL oligonucleotide in 20 μl phosphate buffer (10 mM, pH 7.5) was mixed with 40:1
30 molar excess of the bifunctional crosslinker SMCC in

DMF (30 mM stock solution) and incubated at room temperature for 2 hours. Excess SMCC was removed using a Nick-Spin Column (Amersham-Pharmacia, Orsay, France) which had been equilibrated with PBS. The recovered
5 oligonucleotide solution (100 μ l) was immediately reacted with tenfold molar excess of NLS-peptide (or mutated-NLS-peptide) overnight at room temperature, then stored at -20°C. The oligo-peptide conjugate was purified by preparative PAGE (20% acrylamide denaturing
10 gel containing 8 M urea. Electrophoresis was done 3 hours at 60 W). The coupling yield was 30 %, based on quantification of the radiolabeled oligonucleotides after migration in a 20% denaturing polyacrylamide gel. To assess the peptide content, 1 pmol of radiolabeled
15 oligo-NH₂ or oligonucleotide-NLS was incubated in 10 mM Tris-HCl pH 8, 1 mM EDTA in the presence of proteinase K (0.5 mg/ml) for 10 min at 37°C, followed by incubation for 10 min at 65°C and subsequent loading on a denaturing polyacrylamide gel. Visualization and
20 quantification were performed with a PhosphorImager 425 (Molecular Dynamics, SA).

iii) Ligation of the CMVLuc restriction fragment to the hairpin oligonucleotides

25 pCMVLuc plasmid, encoding the *Photinus pyralis* luciferase under the control of the cytomegalovirus enhancer/promoter and followed by the SV40 early polyadenylation signal was prepared as described in WO 93/07283 (designated there "pCMVL"), and propagated
30 and purified as described (Zanta et al., 1997).

*Xmn*I/*Xma*I double digestion (10 U/ μ g DNA) was performed at 37 °C for 2 hours, followed by enzyme heat inactivation (65°C for 20 min), prior to *Sal*I cleavage (37°C for 2 hours; 10 U/ μ g DNA). After removal of the
5 endonucleases by DNA precipitation, *Bsp*HI digestion was performed for 2 hours at 37°C (10 U/ μ g DNA). Separation of the CMVLuc-containing DNA fragment (3380 bp) from the shorter restriction fragments was performed by ultracentrifugation (30,000 rpm, 19 hours at 25°C,
10 Beckman Ultracentrifuge L8/55, France) in a 15-30% sucrose gradient.

The 5'-end of the oligo-peptide carrying the NLS or the mutated-NLS peptide was radiolabeled with [γ -³²P]-ATP and T4 polynucleotide kinase (1 U/pmol oligonucleotide
15 at 37°C for 30 min). The 5'-end of the oligo-cap was phosphorylated similarly with ATP. Excess [γ -³²P]-ATP and ATP were removed using Microspin G-25 columns (Amersham-Pharmacia). Prior to ligation, the hairpin form of the oligonucleotides presenting a sticky 5'-end
20 was obtained by boiling and subsequently cooling the sample in ice. Ligation of the CMVLuc fragment with the oligo-cap and the oligo-peptide was performed overnight at 13 °C with a 15-fold molar excess of each oligonucleotide and T4 DNA ligase (10,000 U/ μ g DNA).
25 The excess oligonucleotide was removed using a Microspin S-400 HR column (Amersham-Pharmacia). Quantification of the ligase reaction yield (ca. 80-90 %) was performed by Cerenkov counting (TRI-CARB 2100 TR Liquid Scintillation Analyzer, Packard,
30 France). Capping of the CMVLuc fragment was checked by digestion with Exonuclease III (10 U/ μ g DNA) at 37°C. Agarose gel electrophoresis showed the uncapped and

hemicapped fragments to be totally digested, whereas the capped fragment remained undigested.

If not stated otherwise, the following materials and methods were used in the Experiments described below:

5 i) Cells and cell culture

NIH 3T3 murine fibroblasts were purchased from ATCC (Rockville, MA, USA) and grown in DMEM (Gibco BRL, Cergy-Pontoise, France). BNL CL.2 murine hepatocytes (ATCC) were grown in DMEM high glucose (4.5 g/l). HeLa
10 human cervix epitheloid carcinoma cells were grown in MEM with Earle's salt (PolyLabo, Strasbourg, France). Human monocyte-derived macrophages, from a healthy donor (Hautepierre Hospital, Strasbourg) and isolated from blood by Ficoll, were grown in RPMI 1640
15 (Biowhittaker). Dorsal root ganglia (DRG) new born rat neurons were obtained and grown as described by Lambert et al., 1996. All cell culture media were supplemented with 10% FCS (fetal calf serum, Gibco BRL), 2 mM L-glutamine, 100 units/ml penicillin and 100 µg/ml
20 streptomycin (Gibco BRL). Cells were maintained at 37°C in a 5% CO₂ humidified atmosphere.

ii) Cell transfection

For each cell line used, 10,000 cells/well were seeded twenty four hours before transfection in 96 multi-well
25 tissue culture plates (Costar, D. Dutscher, France) in order to reach 60-70% confluence during transfection. Before transfection, cells were rinsed and 0.2 ml of fresh culture medium supplemented (transfection in the presence of serum) or not (transfection in the absence

of serum) with 10% FCS was added to each well. The desired amount of DNA was diluted into 46 μ L (final volume) of 0.15 M NaCl or 5% glucose solutions. The desired quantity of ExGen500, 25 kDa PEI or Transfectam
5 (from a 1 mM aqueous amine nitrogen stock solution of PEI or a 2 mM ethanolic stock solution of Transfectam) was then added to the DNA-containing solutions, vortexed gently and spun down. After 10 min, volumes corresponding to 10, 20 or 200 ng of CMVLuc fragment
10 (10 ng/ μ l DNA) or to the gene-number corrected amount of plasmid DNA were added to the cells. The cell culture dish was immediately centrifuged (Sigma 3K10, Bioblock, France) for 5 min at 1500 rpm (280 g) or 500 rpm for primary neurons. After 2-3 hours, 20 μ l of
15 fetal calf serum were added to the serum-free wells. Cells were cultured for 24 hours and tested for reporter gene expression. All experiments were done in duplicate.

iii) Luciferase Assay

20 Luciferase gene expression was measured by a luminescence assay. The culture medium was discarded and cell lysate harvested following incubation of cells for 30 min at room temperature in 100 μ l of Lysis Reagent 1x (Promega, MA, USA). The lysate was vortexed
25 gently and centrifuged for 5 min at 14,000 rpm at 4°C. Twenty μ l of supernatant were diluted into 100 μ l of luciferase reaction buffer (Promega) and the luminescence was integrated over 10 seconds (Mediators PhL, Wien, Austria). Results were expressed as light
30 units per mg of cell protein (BCA assay, Pierce).

Example 2

Synthesis of a capped gene-peptide conjugate ;
purification and proof of structure

The construction was based on ligation of a pair of
5 hairpin oligonucleotides to unique cohesive termini
generated on the reporter gene, as schematically
depicted in Fig. 1. Incorporation of ^{32}P into the
modified oligonucleotide allowed us to follow the
reaction kinetics, to purify the fragment of interest
10 and to verify its structure. The firefly luciferase
reporter gene (Luc) flanked by the cytomegalovirus
(CMV) enhancer/promoter sequence and the SV40
polyadenylation signal was excised from the pCMVLuc
plasmid (Fig. 1). Quadruple endonuclease digestion
15 ensured straightforward large-scale separation of the
3380 bp CMVLuc fragment from the remaining < 1 kbp
fragments by ultracentrifugation through a 15-30%
sucrose gradient. T_4 -loops are well-suited for hairpins
and the C-terminal thiol group of the PKKKRKVEDPYC
20 peptide was conjugated to a thymine with a C(5)-amino
group (Seibel et al., 1995) via an activated
ester/maleimide bifunctional linker (SMCC) as detailed
in Fig. 2: A hairpin oligonucleotide with a free
alkylamino group in the T_4 loop (oligo- NH_2) was reacted
25 with the heterobifunctional crosslinker SMCC to give a
thiol-reactive maleimide oligonucleotide (oligo-mal)
which was in turn reacted with the C-terminal
cysteinamide residue of the NLS dodecapeptide).

Preliminary experiments showed that the global reaction
30 yield was highest following activation of oligo- NH_2 with

SMCC for 2 h (significant parasitic maleimide hydrolysis may occur with time). Oligonucleotides 5'-³²P radiolabeling and polyacrylamide gel electrophoresis (Fig. 3A: Polyacrylamide gel electrophoresis (PAGE) analysis of the formation of the oligo-peptide conjugate. Aliquots of the reaction mixture were taken at different time intervals and analyzed on a 20% denaturing gel after radiolabeling of the oligonucleotides. Lane 1 : oligo-NH₂; lane 2: oligo-NH₂/SMCC reaction mixture after 2 h; lanes 3, 4 and 5: reaction mixture 1.5 h, 3 h or overnight after addition) showed the peptide conjugation reaction to be completed after 3 h, giving 30% oligonucleotide-NLS based on the starting oligonucleotide. Proteinase K digestion converted oligonucleotide-NLS to a faster migrating compound, presumably the oligonucleotide conjugated to the C-terminal aminoacid (oligo-mal-cys), thus establishing the chimeric nature of the conjugate (Fig. 3B: Radiolabeled oligo-NH₂ or the crude oligonucleotide-NLS reaction mixture were digested with proteinase K. Products were analyzed on a 20% denaturing gel and show total conversion of oligonucleotide-NLS into a faster migrating band, presumably oligo-mal-cys). The oligonucleotide-NLS was purified by electrophoresis. The uncapped CMVLuc fragment was then simultaneously reacted with a 15-fold excess of oligo-cap and oligonucleotide-NLS using T4 DNA ligase in previously optimized conditions. Ligation reaction yield (80-90%) was assessed by quantitative radioactivity counting and full capping of the CMVLuc fragment was checked by 3'-exonuclease digestion. CMVLuc-NLS was purified by gel permeation.

Example 3

Transfection with low amounts of DNA

The reporter gene construct was obtained by chemical/enzymatic synthesis and purified by PAGE/gel permeation, instead of being amplified in bacteria. Only limited amounts of DNA could therefore be obtained and the transfection setup had to be miniaturized. A convenient 96-well microtiter plate assay developed by Felgner et al., 1994, was chosen. Using several optimized cationic lipid formulations, these authors showed that transfection was best at 2-0.5 μ g plasmid and quickly fell off below 0.25 μ g. This result was confirmed with pCMVLuc and two other cell types (Table 1, entry 4 and Fig. 4), thus putting our ultimate goal to obtaining an effective transfection with less than 200 ng DNA. As a preliminary test, the relevance to cap the reporter gene at both ends was investigated, thus avoiding free DNA ends that are prone to exonuclease-mediated DNA degradation and to eliciting a DNA repair response. To this end, the uncapped, hemicapped and fully capped CMVLuc DNAs (200 ng) were transfected into hepatocytes. (BNL CL.2 hepatocytes were transfected with 200 ng DNA/well. Complexes were formed with 25 kDa PEI (N/P= 10) in 150mM NaCl, and in the absence of serum.)

Luciferase activities (Table 1) showed both un- and hemi-capped molecules to give 25-fold less gene expression than the capped one thus justifying the choice of our chemical strategy. Thus, this is experiment shows that free DNA ends decrease

transfection efficacy. (a: Transfection with 20 ng plasmid.)

Example 4

5 The NLS peptide allows effective transfection with minute quantities of DNA

3T3 cells were transfected with decreasing amounts of DNA, using the NLS-bearing capped gene (CMVLuc-NLS), the capped gene (CMVLuc) and the corresponding mass-corrected amount of plasmid DNA (pCMVLuc). While efficacy very much decreased for '20 ng' plasmid DNA (in fact 38 ng), as expected (see above), transfection levels remained remarkably high and constant over the 3T3 cells were transfected with decreasing amounts of DNA complexed in 150 mM NaCl to a cationic lipid (Transfectam, N/P=6) or to a cationic polymer (25 kDa PEI N/P=10) in the absence of serum. range 200-10 ng DNA with CMVLuc-NLS (Fig. 4: 3T3 cells were transfected with decreasing amounts of DNA complexed in 150 mM NaCl to a cationic lipid (Transfectam, N/P=6) or to a cationic polymer (25 kDa PEI N/P=10) in the absence of serum.). Comparison with the capped gene lacking the NLS peptide showed 100-1000 fold more expression when the nuclear localization signal peptide was present on the gene. Similar conclusions were obtained, whether using a cationic lipid (Transfectam, Fig. 4 left) or a cationic polymer (branched PEI, Fig. 4 right). Further data (not shown) confirmed that other cationic carrier molecules behaved similarly, but also that efficacy dropped fiftyfold within the range 10-1 ng CMVLuc-NLS.

Improved transfection involves the cellular nuclear import machinery. Comparative experiments between CMVLuc and CMVLuc-NLS, although interesting, do not really allow one put forward any hypothesis concerning the mechanism. Nuclear import of DNA and oligonucleotides has mainly been followed with fluorescent tags and FISH, in microinjected or digitonin-permeabilized cells. Proof of active import relied on inhibition experiments using energy-decoupling molecules, nuclear pore-binding agglutinins or mutations in the signal peptide sequence. Gene expression is an unambiguous proof of nuclear localization and transfection can be regarded as a straightforward way of introducing DNA into intact cells. The Lysine \Rightarrow Threonine mutation abolishes nuclear import (Kalderon et al., 1984). Therefore, a capped linear DNA molecule was synthesized (CMVLuc-mNLS) with a mutated PKTKRKVEDPYC sequence. Comparative transfection experiments with HeLa cells are shown in Fig. 5 (HeLa cells were transfected with various amounts of DNA complexed to ExGen500 PEI (N/P=5 in a 5% glucose solution) in the presence of 10% serum.). Irrespective of the amount of DNA used, the Lys \Rightarrow Thr mutation brought transfection down to the level obtained with the capped gene having no peptide at all, confirming the functionality of the NLS/importin- α interaction.

Example 5

NLS peptide-mediated transfection enhancement is a general phenomenon

A series of comparative experiments was undertaken with various cell types. Transfection enhancement due to the presence of the NLS peptide was always observed (Table 2, which shows the average enhancement of transfection with CMVLuc-NLS. Values refer to transfection with 10-20 ng DNA/well, in the absence of serum. a. Estimated spread \pm 30%; b. In the presence of 10% serum during transfection; c. In 24-well plates using 200 ng DNA in the presence of 10% serum.)

However enhancement factors were spread widely with cell type (10-1000-fold); with no obvious relation with tissue origin nor cell type (primary vs. transformed cells). Nondividing primary cells such as human monocyte-derived macrophages and rat dorsal root ganglia neurons showed less impressive enhancement than 3T3 and HeLa cells. However, similar values were also seen for the easily transfected and fast dividing rat hepatocyte-derived cell line BNL CL.2.

The general experimental setup for transfection included 96-well microtiter plates and no serum during 2 hours following addition of the DNA/vector complexes to the cells. Several experiments were also performed on a larger scale (24-well plates) or in the presence of 10% serum during transfection. Table 2 shows the average enhancement of transfection with CMVLuc-NLS. (Values refer to transfection with 10-20 ng DNA/well, in the absence of serum. a: estimated spread \pm 30%;

b: in the presence of 10% serum during transfection;
c: in 24-well plates using 200 ng DNA in the presence
of 10% serum.). The results obtained (Table 2, b and c)
confirmed the general conclusions derived with the
5 96-well setup.

Example 6

Time course of the transgene expression

Active transport of the reporter gene via the nuclear
10 import machinery could result in faster appearing
expression. More interesting, a lower intracellular
barrier to gene delivery means less DNA/vector
complexes required in the cytoplasm, hence lower
toxicity and more sustained expression. The kinetics of
15 gene expression was followed in detail up to 24 h
(Fig. 6: HeLa cells were transfected with 10 ng DNA
complexed to ExGen 500 PEI (N/P=5 in 5% glucose
solution) in the absence of serum. Cells were lysed and
luciferase activity was measured at the indicated time
20 after transfection. The absence of error bars indicates
errors that are smaller than the label on the graph.)
and then daily up to 3 days. Again, transfection with
CMVLuc-NLS was much more effective than with CMVLuc-
mNLS. Remarkably (and reproducibly), NLS-driven
25 transfection reached its plateau after 12 h, whereas
transfection levels obtained with the plasmid or the
mutated NLS sequence were still increasing
significantly up to 24 h. Previous experiments (Pollard
et al., 1998) showed both transfection and cytoplasmic
30 injection of DNA/PEI complexes to have superimposable

kinetics of transgene expression, suggesting that the slow step was intracellular trafficking/nuclear entry rather than cell entry. Using the nuclear import machinery seems to speed up the rate-limiting step. For longer time periods, expression stayed broadly constant with a low standard deviation. However, the rather large variability observed upon repeating this experiment prevented any conclusion from being drawn (the inconsistent results observed at $t > 1d$ may be due to the fact that cells reached confluency at day 1).

Table 1

5


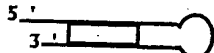
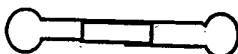

DNA form	Symbol	Transfection efficiency (RLU/mg of protein)
Uncapped CMVLuc		$1 \pm 0.4 \times 10^5$
Hemi-capped CMVLuc		$9 \pm 2 \times 10^4$
CMVLuc		$2.5 \pm 0.6 \times 10^6$
Circular pCMVLuc plasmid		$2.5 \pm 0.2 \times 10^8$ $3 \pm 1 \times 10^4$ a

Table 2

5

CELL TYPE	Enhancement factor ^a
BNL CL.2	10
3T3	400 ^b - 1000
HeLa	200 ^c - 300
Murine DRG neurons	30
Human macrophages	10

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Claims

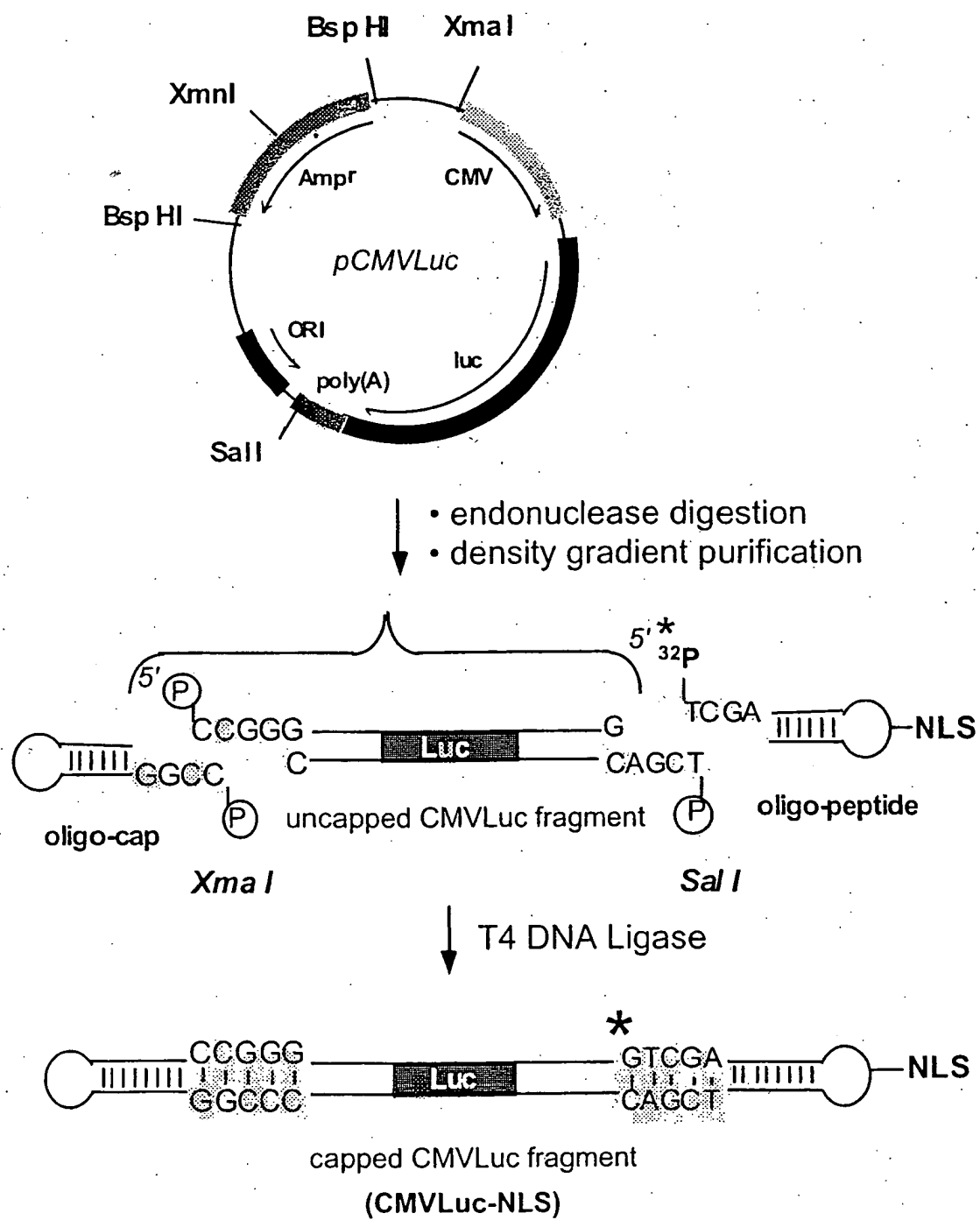
1. A non-viral transfection vector comprising a DNA
5 molecule which is to be delivered into the nucleus
of a cell, characterized that said DNA molecule is
equipped with 1 to 25 conjugates comprising a
nuclear localization signal (NLS) covalently linked
to an oligonucleotide, each of which NLS conjugate
10 has been
 - i) covalently linked to one or both termini of a
linear DNA molecule, or
 - ii) associated with a plasmid DNA molecule by
15 forming a triple helix, or
 - iii) inserted in a plasmid DNA molecule by strand
invasion.
2. The transfection vector of claim 1, wherein said
DNA molecule is equipped with 1 to 10 NLS
20 conjugates.
3. The transfection vector of claim 2, wherein said
DNA molecule is equipped with 1 to 5 NLS
conjugates.
4. The transfection vector of claim 1, characterized
25 in that the linear DNA molecule defined in i) is
equipped, at both ends, with an oligonucleotide
hairpin, one or both hairpins being conjugated to
an NLS peptide.
5. The transfection vector of claim 4 which contains a
30 single NLS peptide.

6. The transfection vector of claim 1, wherein the plasmid DNA molecule defined in ii) is associated with an NLS conjugated to an oligonucleotide which recognizes a homopurine or a homopyrimidine sequence within the plasmid in a sequence specific manner.
7. The transfection vector of claim 6, wherein the oligonucleotide is a homopurine.
8. The transfection vector of claim 1, wherein the plasmid DNA defined in iii) is equipped with an NLS conjugated to an oligonucleotide that is chemically modified such that the D-loop formed through strand invasion is stabilized.
9. The transfection vector of claim 8, wherein the oligonucleotide is modified with spermine.
10. A pharmaceutical composition containing, as an active ingredient, a transfection vector of claim 1, wherein said DNA molecule encodes a therapeutically active protein.
11. A method of transfecting a cell with a DNA molecule, wherein the cell is contacted with a transfection vector defined in claim 1.
12. A method of transfecting a cell with a DNA molecule, wherein the transfection vector defined in claim 1 is complexed with a cationic compound and the cells are contacted with the complex.
13. The method of claim 12, wherein the cationic compound is a cationic lipid.

14. The method of claim 12, wherein the cationic compound is polyethylenimine.

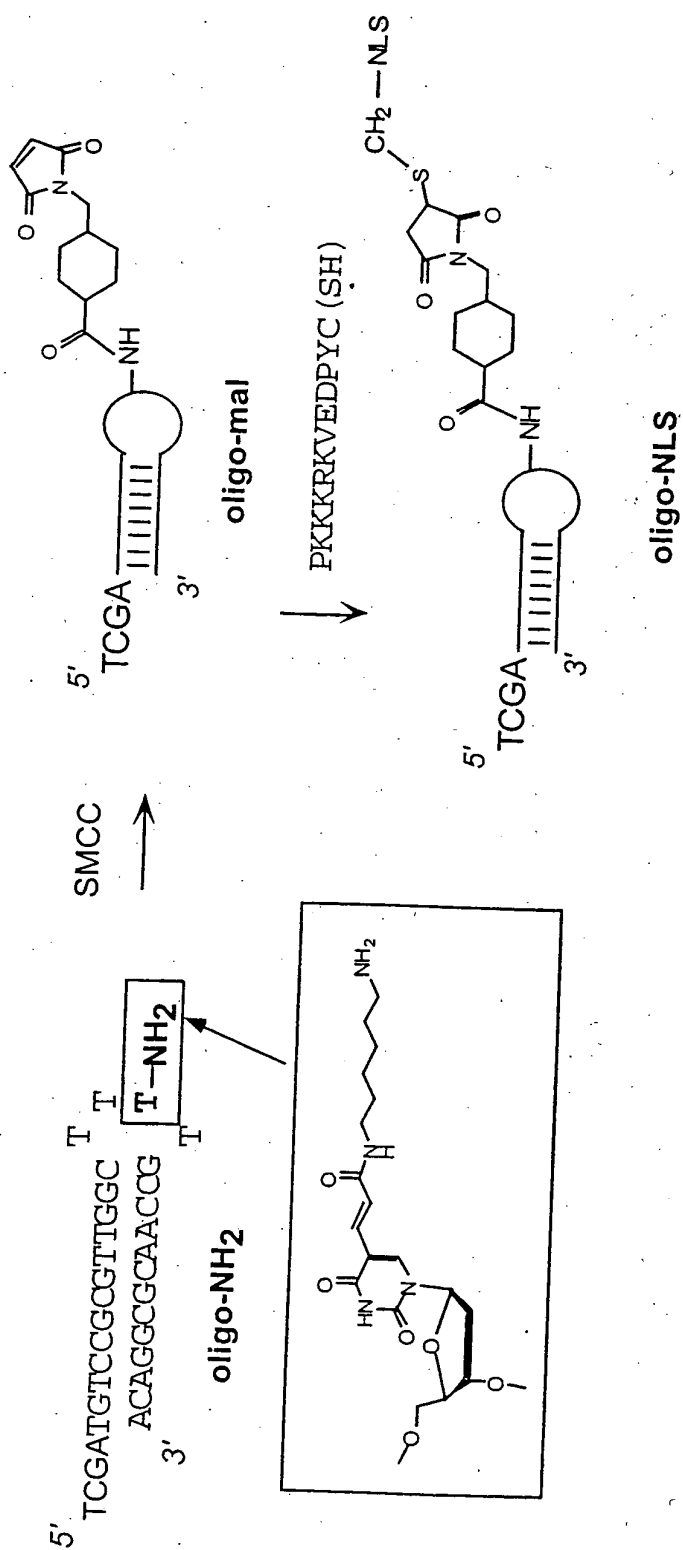
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Fig. 1



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Fig. 2

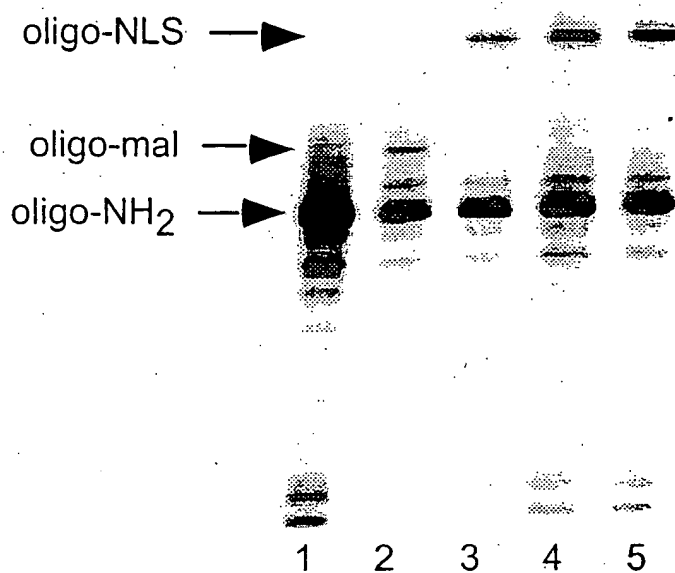


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Fig. 3

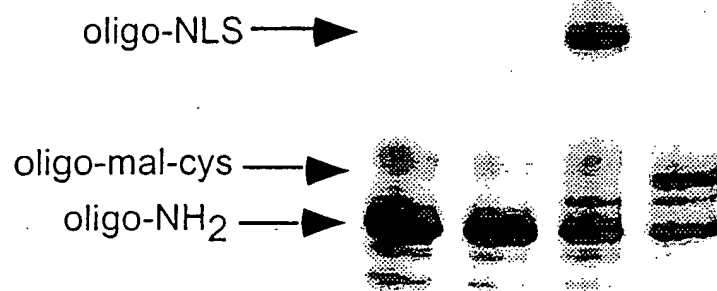
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NLS peptide	-	-	+	+	+
SMCC	-	+	+	+	+
oligo-NH ₂	+	+	+	+	+
		0	1.5 h	3h	o.n.



B

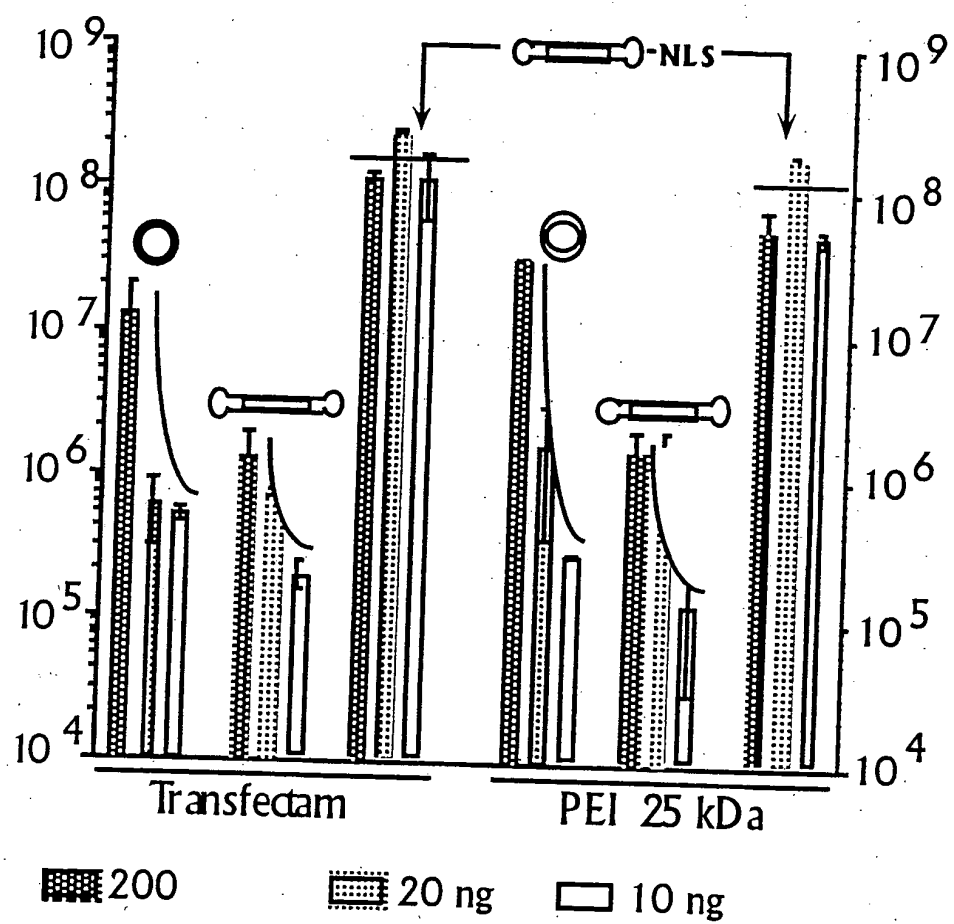
	oligo-NH ₂	reaction mixture
Prot K	-	+
	-	+



SUBSTITUTE SHEET (RULE 26)

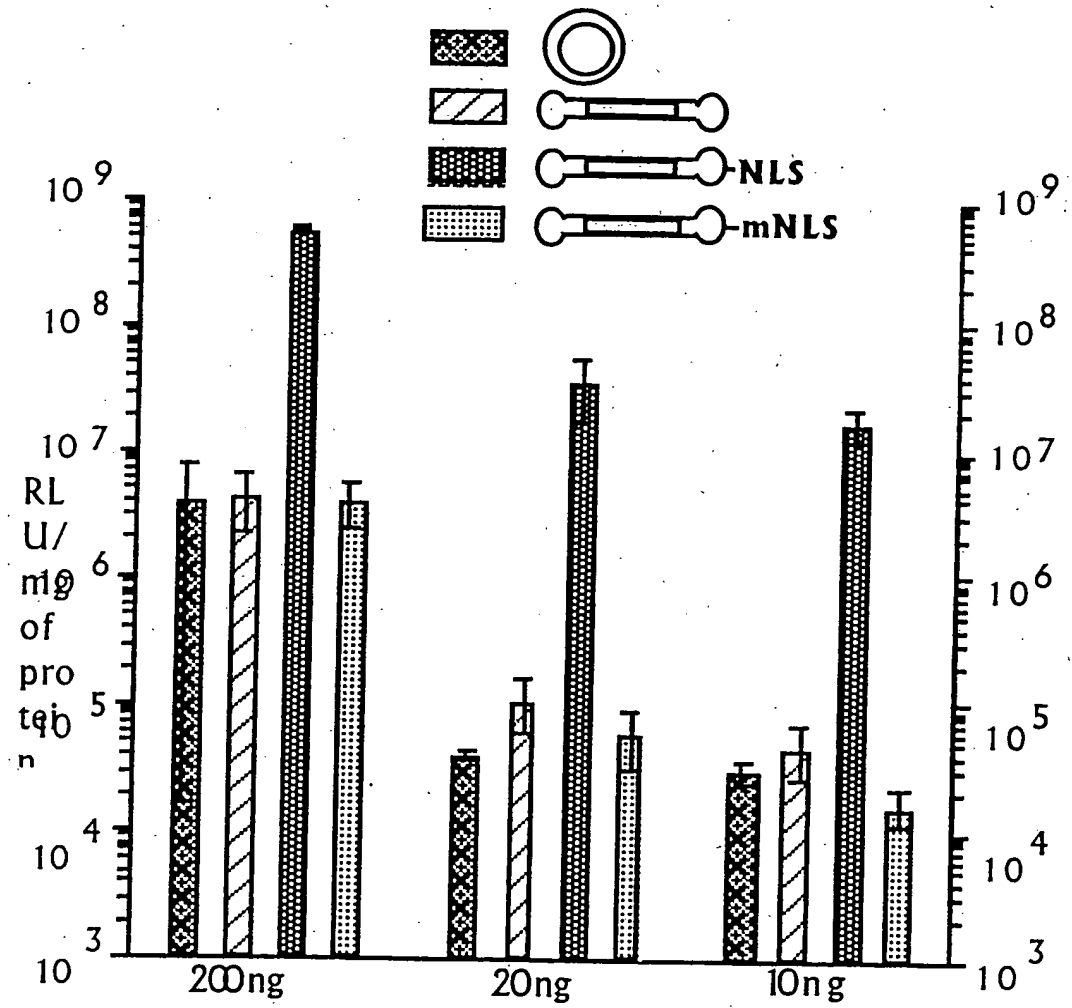
4/12

Fig. 4



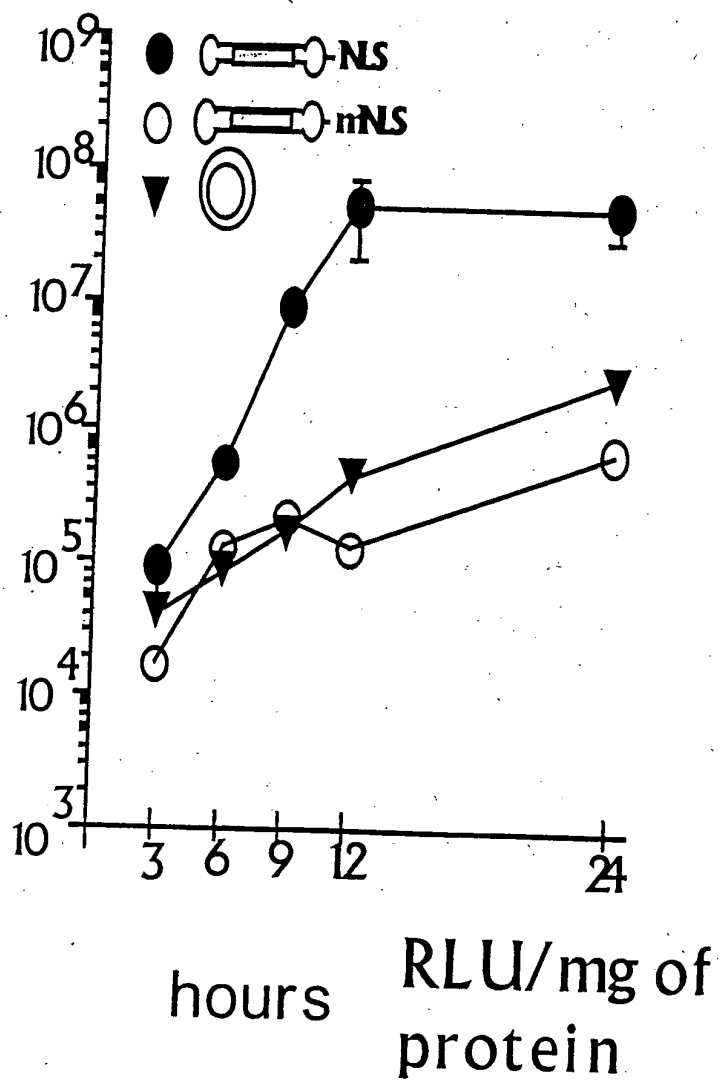
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Fig. 5



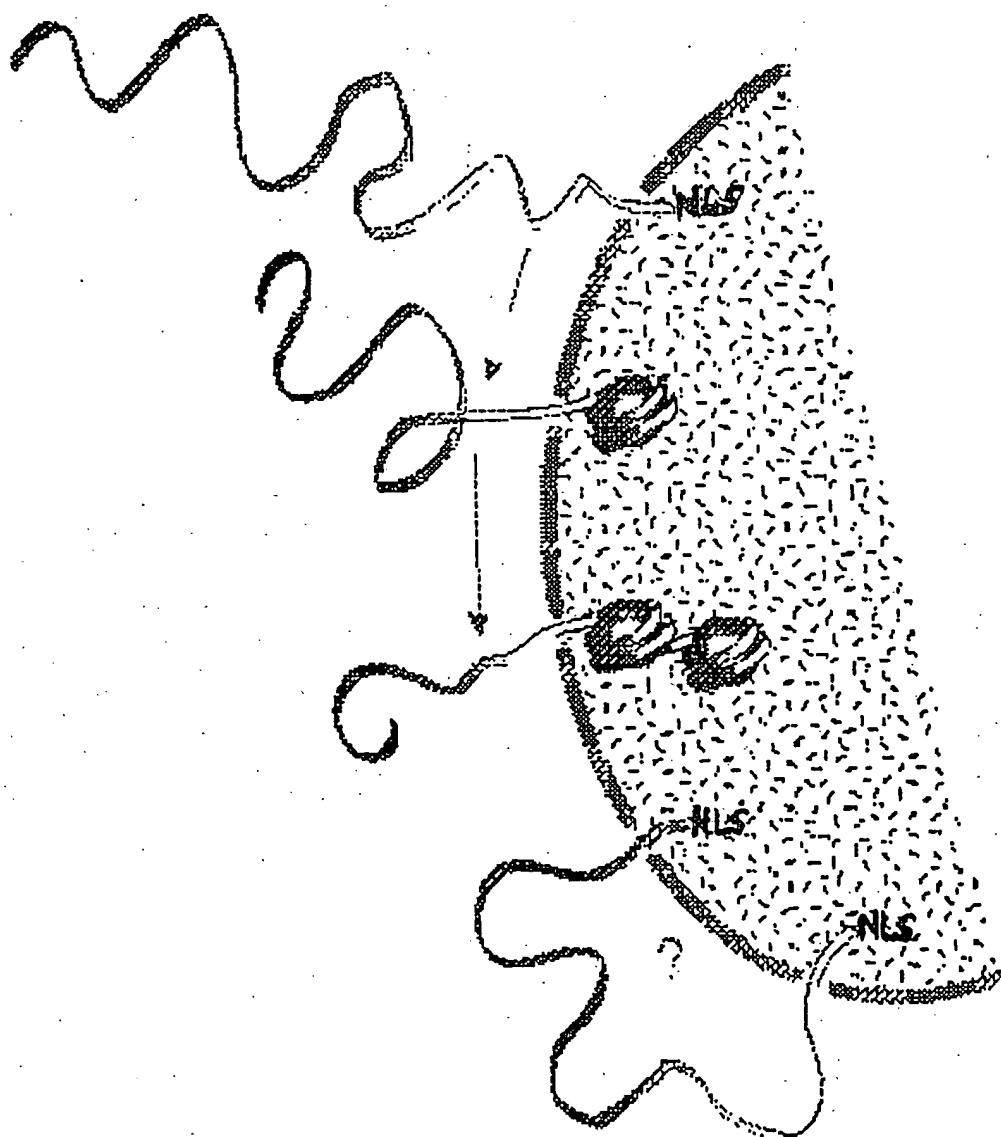
6/12

Fig. 6



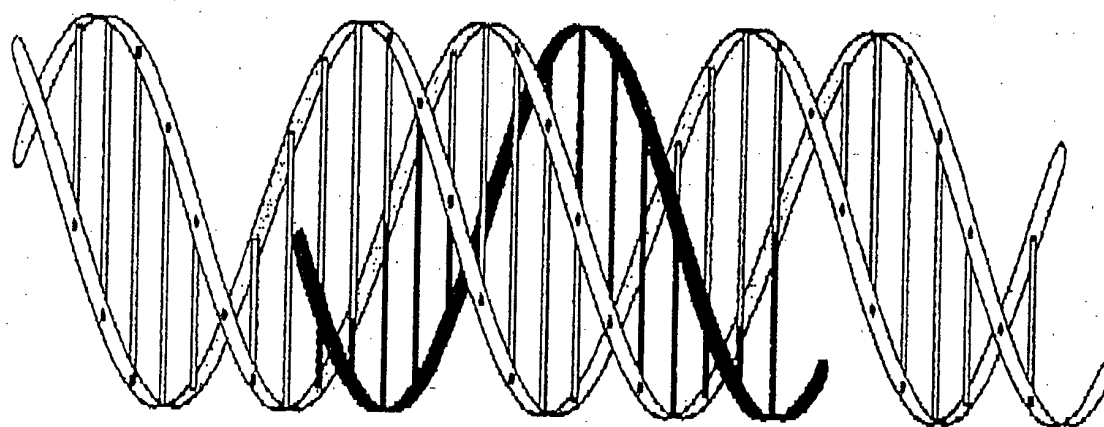
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Fig. 7



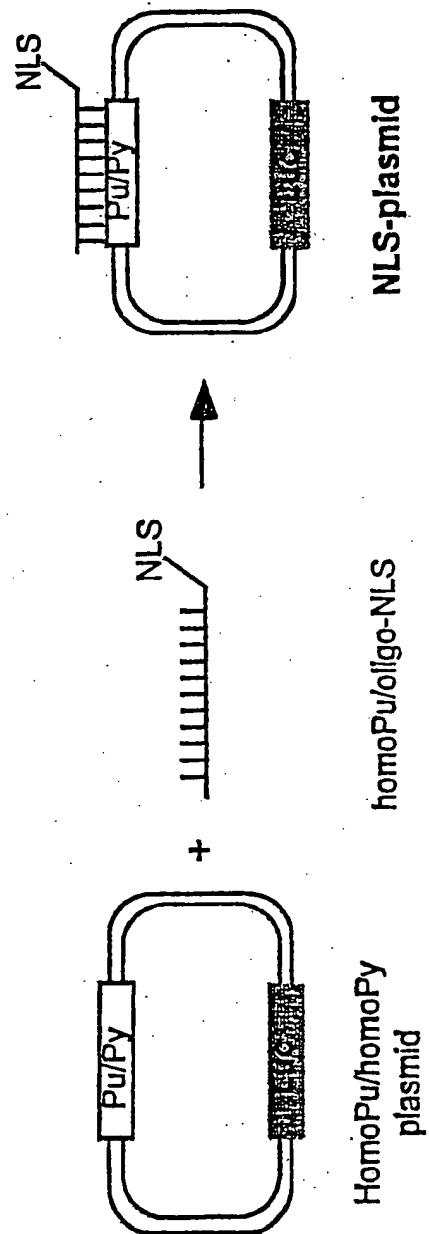
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Fig. 8



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Fig. 9



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Fig. 10

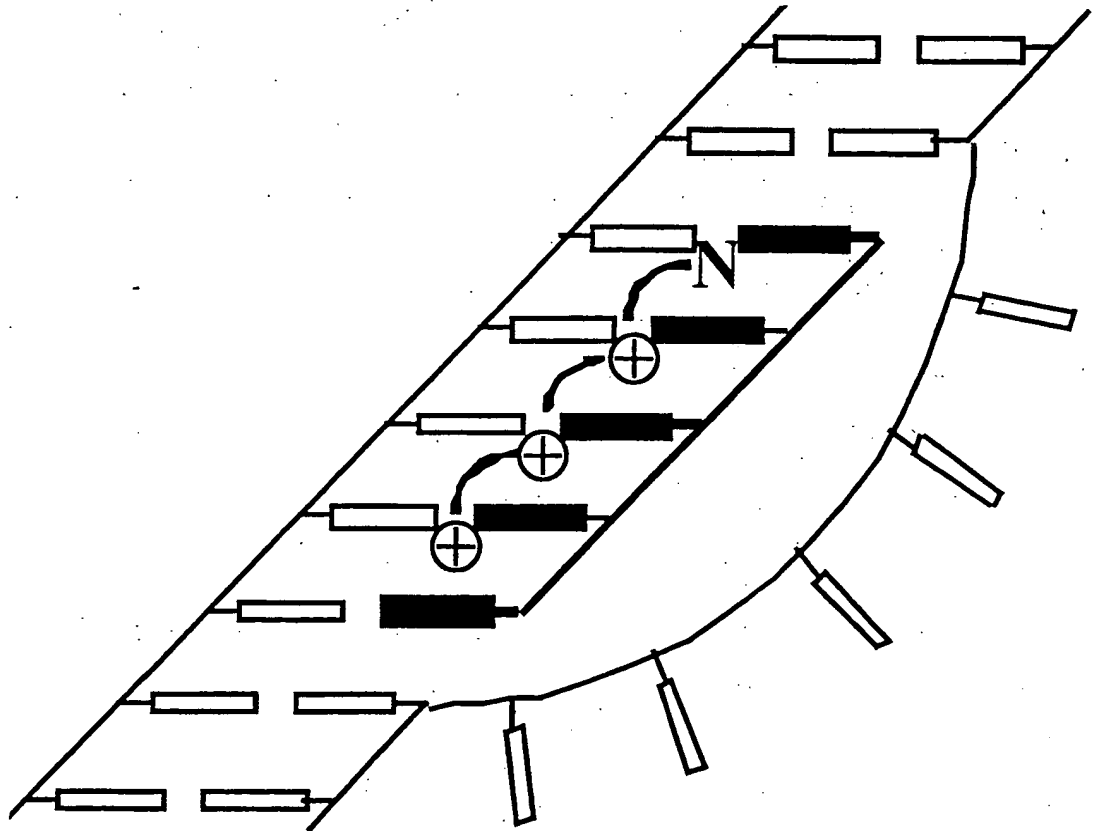
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3' ----AGGGAAGGGAGGAGGAGGGGGAGAGGTAAG---5' human $\alpha 1$
procollagen gene

5' ----CCTCCTCCCCTCCTTCTTCCCCCTTC---3' Friend target
3' ----GGAGGAGGGGAGGAAGAAGGGGGAAG---5'

5' ----CCCCTCCCCCTCC---3' promoter of c-pim-1
3' ----GGGGAGGGGGAGG---5' murine proto-oncogene

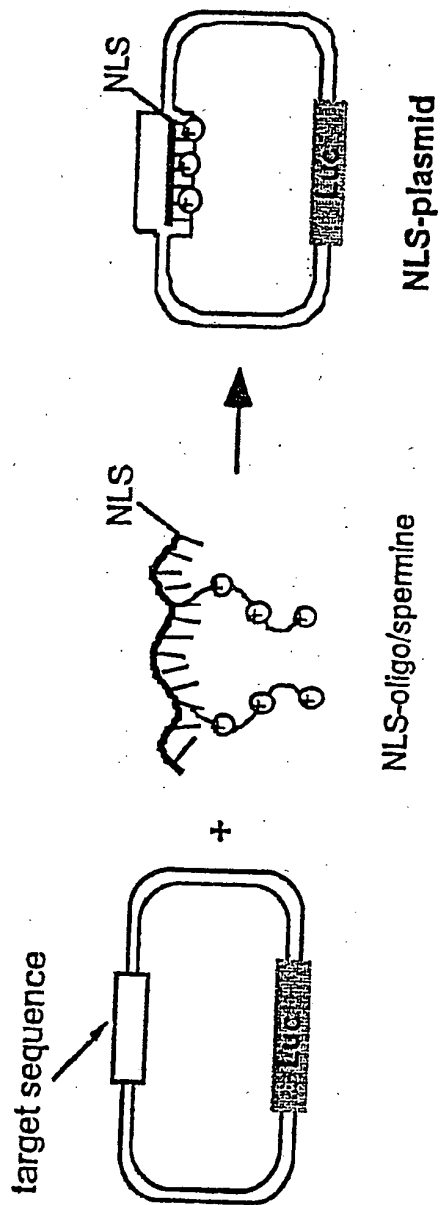
11/12

Fig. 11



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Fig. 12



SEQUENCE LISTING

<110> Université Louis Pasteur de Strasbourg

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 nuclear localization signal peptide

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 Pro Lys Thr Lys Arg Lys Val Glu Asp Pro Tyr Cys
 1 5 10

INTERNATIONAL SEARCH REPORT

International Application No.

PCT/EP 99/10281

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/79 C12N15/85

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	SEBESTYEN MG ET AL: "DNA vector chemistry: the covalent attachment of signal peptides to plasmid DNA" NATURE BIOTECHNOLOGY, vol. 16, 1 January 1998 (1998-01-01), pages 80-85, XP002089214 cited in the application the whole document	
A	WO 95 31557 A (CLONEXPRESS INC) 23 November 1995 (1995-11-23) cited in the application the whole document	
	-/--	

☒ Further documents are listed in the continuation of box C.☒ Patent family members are listed in annex.

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"S" document member of the same patent family

Date of the actual completion of the international search

6 Apr11 2000

Date of mailing of the international search report

13/04/2000

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Oderwald, H

INTERNATIONAL SEARCH REPORT

Inter national Application No
PCT/EP 99/10281

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	DAMHA M J AND NORONHA A: "Recognition of nucleic acid double helices by homopyrimidine 2',5'-linked RNA" NUCLEIC ACIDS RESEARCH, vol. 26, no. 22, November 1998 (1998-11), pages 5152-5156, XP002104947 the whole document	
A	SCHMID N AND BEHR J-P: "Recognition of DNA sequences by strand replacement with polyamino-oligonucleotides" TETRAHEDRON LETTERS, vol. 36, no. 9, 1995, pages 1447-1450, XP002104948 cited in the application the whole document	
P,X	ZANTA M A ET AL: "Gene delivery: a single nuclear localization signal peptide is sufficient to carry DNA to the cell nucleus." PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES OF AMERICA, (1999 JAN 5) 96 (1) 91-6. JOURNAL CODE: PV3. ISSN: 0027-8424., XP002104949 the whole document	1-14

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/EP 99/10281

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9531557 A	23-11-1995	US 5670347 A	23-09-1997
		CA 2189975 A	23-11-1995
		EP 0759996 A	05-03-1997
		JP 10500022 T	06-01-1998

